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Identification of intimin alleles in pathogenic Escherichia coli by PCR-restriction fragment length polymorphism analysis

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Abstract
A rapid two-step identification method based on PCR-RFLP analysis of the intimin gene was developed to differentiate specific alleles in pathogenic Escherichia coli. This technique, tested on isolates eae-positive, accurately detects eae and resolves alleles encoding the α1, α2, β, γ1, γ2/θ, κ, ε, ζ, and ι intimin variants.

Keywords :
Escherichia coli . Intimin . PCR-RFLP

Abbreviations :
A/E attaching and effacing
EHEC enterohaemorrhagic E. coli
EPEC enteropathogenic E. coli
LEE locus of enterocyte effacement
PCR polymerase chain reaction
RFLP restriction fragment length polymorphism

Introduction

Enteropathogenic Escherichia coli (EPEC) and enterohaemorrhagic E. coli (EHEC) produce the characteristic "attaching and effacing" (A/E) lesion in the gut mucosa of humans and animals. The A/E lesion is due to marked cytoskeletal changes and is characterised by effacement of microvilli and close adherence between the bacteria and the ephitelial cell membrane, with accumulation of polymerised actin directly beneath the adherent bacteria (Nataro and Kaper 1998). Production of A/E lesions is associated with the expression of intimin, an outer membrane protein encoded by a gene (eae) which is part of the LEE (locus of enterocyte effacement) pathogenicity island (Elliott et al. 1998; Nataro and Kaper 1998).The detection of the eae gene was taken as an indicator for the presence of the A/E pathogeneticy factor in a strain (Knutton et al. 1991) and different genetic variants of the eae gene were described during time (Lacher et al. 2006; Tarr and Whittam 2002; Zhang et al. 2002). In a recent study (Lacher et al. 2006), a fluorescent RFLP (fRFLP) method was reported to identify many allelic variants of eae locus and can reveal previously unknown allelic variants.
In the present study we propose a PCR-restriction fragment length polymorphism analysis (RFLP) assay for simultaneous and rapid detection of the eae gene and alleles encodind the α1, α2, β, γ1, γ2/θ, κ, ε, ζ, and ι intimin variants in the E. coli genome.

Materials and methods

The E. coli reference strains (Oswald et al. 2000; Zhang et al. 2002) which were used as positive controls to design, and optimize the PCR-RFLP assay are described in Table 1. Once set up, PCR-RFLP was tested on 20 eae-positive E. coli strains isolated in Italy from feces of cattle. These strains, had been previously analysed with universal eae primers SK1 and SK2 and subtyped with primer SK1 in combination with LP2, LP3, LP4, LP5, LP6B, LP7, LP8, LP10, and LP11 (Oswald et al. 2000; Zhang et al. 2002). PCR assays led to the identification of 7 eae-genotypes: β (7 strains), θ (4 strains), α (3 strains), ε (2 strains), γ (2 strains), κ (1 strain) and ι (1 strain). Intimin subtypes ζ and η were not found.
In preparation of PCR-RFLP, each E. coli strain was grown overnight at 37°C in Luria broth. The primer sequences were designed to amplify a 1,300 bp fragment within the coding region of the intimin gene in E. coli strains. The design was based on an alignment of the published eae sequences of E. coli strains, which demonstrated common regions serving as targets for the primers. Variable regions between these targets suggested beforehand restriction enzyme differentiation. The chosen restriction enzyme exploited these variable regions. The forward primer eae-F (5′-AGGATATTCTTTCTCTGAATA-3′) was constructed in the N-terminal conserved region of the gene, whereas the reverse primer eae-R (5′-ATATYTATTTGCWGSVCCCCAT-3′) was constructed in the variable C-terminal region of the protein. Amplification was performed in a 50 µl reaction volume containing 5 µl of DNA sample, 50 pmol of each

primer (Sigma-Genosys, Cambridge, UK), 0.1 mM each dATP, dCTP, dGTP, and dTTP (Amersham Biosciences, Europe, GmbH), PCR buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl), 2.5 U Taq DNA polymerase (Qiagen, Hilden, Germany). The samples underwent an initial denaturation for 3 min at 94°C, followed by 30 amplification cycles, each consisting of 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s. A final primer extension at 72°C for 5 min was included. PCR products were visualized on ethidium bromide (5 μl/ml) stained gels by trans-illumination with UV light and were purified using Kit QiAquick PCR purification (Qiagen).

Table 1 Intimin alleles and PCR-RFLP patterns of reference strains

Intimin allele	Strain name (serotype)	Reference[a]	RFL pattern (band sizes [bp])
			*Msp*I
α 1 (alpha 1)	E2348/69 (O127:H6)	Oswald et al. 2000	350, 950
α 2 (alpha 2)	EF 73 (O125:H6)	Oswald et al. 2000	7, 234, 346, 713
β (beta)	RDEC-1 (O15:H-)	Oswald et al. 2000	130, 450, 722
γ 1 (gamma 1)	EDL933 (O157:H7)	Oswald et al. 2000	460, 840
γ 2 (gamma 2)	95NR1 (O111:H-)	Oswald et al. 2000	190, 1,110
ε (epsilon)	PMK5 (O103:H2)	Oswald et al. 2000	450, 850
ζ (zeta)	4795/95 (O84:H4)	Zhang et al. 2002	530, 770
θ (theta)	CL37 (O111:H8)	Zhang et al. 2002	190, 1110
ι (iota)	7476/96 (O145:H4)	Zhang et al. 2002	590, 710
κ (kappa)	6044/95 (O118:H5)	Zhang et al. 2002	190, 530, 580

[a] First description of the strain's virulence factors

For RFLP analysis, restriction endonuclease was selected from restriction maps of the intimin sequences of the reference strains. Selection criteria were based on the minimum number of enzymes required to produce diagnostic restriction fragment profiles. To satisfy these criteria, the enzyme MspI (HpaII) (Fermentas, Milano, Italy) which recognise the sequences C'CGG was selected. For restriction endonuclease digestion, a 25 μl reaction mixture, which included 5 μl of purified amplicon with 10 U of the restriction endonuclease MspI, was used following manufacturer's recommendations. Eight microliters of each digest was analysed electrophoretically in 2% (wt/vol) agarose (Sigma Chemical Co, St. Louis, MO). The gels were stained with ethidium bromide and viewed on UV transilluminator (Geldoc 2000; Biorad, Milano, Italy).

DNA sequences from all reference strains were edited with BioEdit, version 7.0.5. (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) (Hall 1999). Genetic distances were computed using MEGA 2.1 and were used to construct a neighbor-joining tree with the Tamura-Nei two-parameter distance option (Tamura and Nei 1993). Statistical confidence of the tree's topology was assessed by bootstrap (Felsenstein 1985).

Results and discussion

The ability of the PCR-RFLP to resolve intimin alleles was first tested on 10 known subtypes (α1, α2, β, γ1, γ2/θ, κ, ε, ζ, and ι). Although we did not posses an E. coli isolate containing the recently described eae-η (Zhang et al. 2002), sequence alignments indicate that our method is able to identify the gene for this subtype. The restriction patterns obtained by PCR-RFLP with DNA from reference strains are shown in Table 1 and Fig. 1 MspI differentiated 9 of 10 subtypes, since the sequences of eae-γ2 and eae-θ should be considered one eae variant (γ2/θ) (Zhang et al. 2002) producing the same restriction pattern (190 and 1,110 bp) (Fig. 1).

Fig. 1 Intimin PCR-RFLP patterns observed with MspI. Lanes: M, 100 bp-plus molecular weight marker; α1, EPEC O127:H6; α2, EPEC O125:H6; β, EPEC O15: H-; γ1, EHEC O157:H7; γ2, EHEC O111:H-; θ, EHEC O111: H8; κ, EHEC O118:H5; ε, EHEC O103:H2; ζ, STEC O84:H4; ι, EHEC O145:H4; M1, 50 bp-plus molecular weight marker

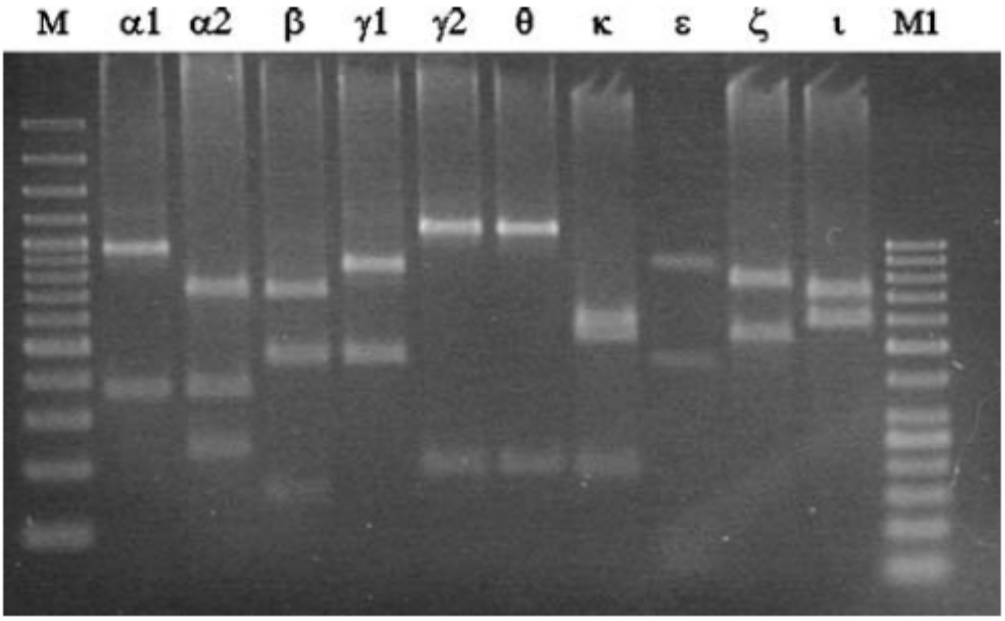
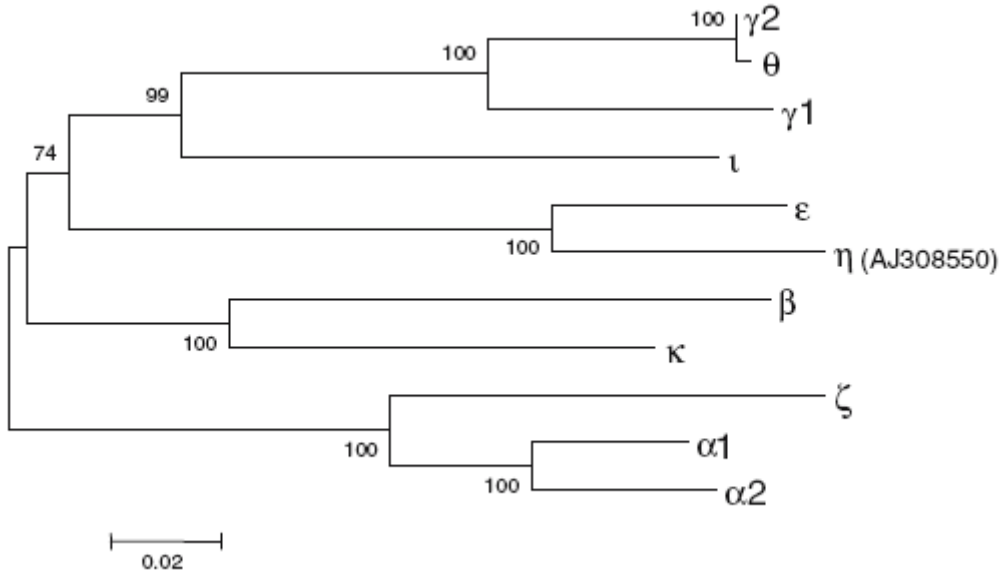


Fig. 2 Phylogenetic tree of sequence similarity among 1300-bp sequences of the eae variants



Results obtained from 20 eae-positive E. coli cattle strains by this method, indicate that all strains possessed the eae gene variants according to data obtained by PCRs. Moreover, the application of PCR-RFLP to E. coli strains, isolated from cattle, show the presence of eae-α1 (2 strains), eae-α2 (1 strains), eae-β (7 strains), eae-γ1 (2 strains), eae-γ2/θ (4 strains), eae-ε (2 strains), eae-κ (1 strain), eae-ι (1 strain). The PCR-RFLP assay was then confirmed by determining the genetic relationship of eae-α1, eae-α2, eae-β, eae-γ1, eae- γ2/θ, eae-κ, eae-ε, eae-ζ, and eae-ι. The 1,300 bp fragments from reference strains were sequenced. Phylogenetic analysis deriving from PCR products and from sequence of gene eae-η (GenBank accession no. AJ308550), showed that sequences grouped in four main clusters, eae-α/eae-ζ, eae-γ1/eae-γ2/θ, eae-ε/eae-η and eae-β/eae-κ. eae-é are closely related to the alleles of the γ group (100% bootstrap support) (Fig. 2), as previously reported by Zhang et al. (2002).

PCR methods using single primer sets have been reported (Oswald et al. 2000; Zhang et al. 2002) and so screening of the eae genes requires a large number of individual PCRs. To reduce the number of tests needed to identify E. coli intimin genes, we have developed PCRRFLP that is able to characterize 9 intimin variant types (α1, α2, β, γ1, γ2/θ, κ, ε, ζ, and ι). Results demonstrate that the PCR-RFLP allowed a rapid genetic identification of the eae alleles and therefore it is relatively simple and highly discriminatory when compared to single PCR protocols on the same targets.

It is also faster and more economic than other techniques, as fRFLP (Lacher et al. 2006), that require expensive equipments and involve complex and time-consuming steps, even if more sensitives. In conclusion our intimin typing method will have medical and veterinary clinical applications for the classification of suspected pathogens into the major clonal groups of EPEC and EHEC.

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